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The New About Congenital Antimicrobial Defense of Some Epithelial Tissues – Vaginal Mucosa and Hair

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1. Introduction

Antimicrobial peptides (AMP) are a family of more than 500 different substances which protect mucosal and dry epithelial surfaces of all multicellular organisms (Bals, 2000; Zasloff, 2002). They are widely dispersed in nature and active against broad spectrum of Gram-positive and Gram-negative bacteria, yeasts, fungi and enveloped viruses, therefore they called "natural antibiotics". Most of AMP are the cationic peptides very diversed with structure but demonstrate an affinity for the negatively charged phospholipids which are present on the outer surfaces of the cytoplasmic membranes of many microbial species. As far as it is difficult for a microbe to change the phospholipid organization of its membrane, resistance to the AMP occurs at levels that are much lower than those observed for conventional antibiotics. Besides the direct antimicrobial function AMP are the inflammation mediators participated in such different processes as proliferation, immune induction, wound healing, cytokines release, chemotaxis, protease-antiprotease balance, redox homeostasis. Different human epithelial locuses are investigated for availability of AMP – respiratory tract, oral cavity, skin, colon, vaginal tract. However some "blank spots" occur until now, for example, to what extent the role of AMP in the defense of vaginal tract is important, and do the AMP take part in the defense of hair?

2. Antimicrobial peptides as factor of local immunity in vulvovaginal candidosis

Vulvovaginal candidosis (**VVC**) affected women of reproductive age, at that acute VVC , which strike up to 75% of women , and chronic recurrent VVC (up to 20%) are distinguished (Fidel Jr, 2007). Factors promoted the VVC development reputed the following: availability of current diseases (infectional, endocrinopathic, autoimmune etc.); mechanic traums; chemical factors – use of corticosteroids, cytostatics, antibiotics, oral contraceptives etc.; pregnancy.

It is known that in systemic candidosis a main protective role belongs to cellular immunity, namely to polymorphonuclear leucocytes; in mucosal candidosis, except VVC, most significant are Th1-cells, circulated in the blood and local (Fidel Jr, 2007). In VVC most

important are reputed the factors of local immunity, among which listed phagocytes and epithelial cells (Nomanbhoy et al, 2002), antibodies (Barousse et al, 2004), cytokines and interferon (Shabashova et al, 2006), concentration of lactate and pH, as well as antimicrobial peptides (AMP) secreted by different leucocytes and epithelial cells (Valore et al, 2002, 2006).

In connection with vaginal protection the following peptides are listed: lactoferrin, calprotectin, secretory leukoprotease inhibitor , cathelicidines, lysozyme and defensins.

In **table 1** features of these substances are summarized: some their chemical properties, localization in human organism, antimicrobial activity spectrum, mechanism of action, and minimal inhibiting concentrations against the *Candida* spp., as well as their concentrations in vaginal secretions of healthy women and in patients with VVC.

Origin of VVC is caused by the decrease of protective function of vaginal epithelium, with combined action of different AMP as the part of the protection. Summarizing the data of table 1 one can conclude that the levels of some AMP somewhat higher in patients with VVC than in healthy women, however researchers till now did not demonstrate exact results, which phase of disease was studied – acute or remission? It is logically to propose that the beginning of acute phase of VVC must be caused by the sudden fall of AMP level, whereas the further development of the disease most likely accompanied with gradual increase of AMP level.

Compare of AMP concentrations, which really can suppress *C. albicans* growth, with AMP levels occurred in vaginal secretions may give the information about some peptide substance. Apparently the most significant may be calprotectin, defensin HNP-1 and in some extent lysozyme. Certain of the AMP known to have a synergistic action, for example, lactoferrin and calprotectin can intensify the effect of each other during the growth inhibition of *C. albicans* (Okutomi et al, 1998).

As far as all of AMP are produced by immune cells it is obviously that decrease of their level must be the result of: 1) decrease of the compounds synthesis in the cells; 2) reduction of quantity of the cells; 3) change the structure of AMP resulting to the loss of activity. Anyway the local cell immunity is primary towards the AMP. Deficit of such effective tools of the first defense line, like AMP, the human organism should compensate by alternative mechanisms of resistance to fungal microflora: activation of phagocytal function and increase of specific immunoglobulines synthesis. Recently was found out that monoclonal antibodies Mab C7 obtained by *C. albicans* mannoprotein not only suppressed the adhesion of yeast cells to different surfaces, but had the direct candidacidal activity (Omaetxebarria et al, 2005). Earlier unknown mechanism of AMP induction in vagina was discovered: cathelicidine hCAP-18, contained at high concentrations, but in inert form in semen plasma, falling in vagina with low pH transformed in its active form (Sorensen et al, 2003).

3. Role of AMP compare to other factors of local immunity in women with vulvovaginal candidosis

Reasons of frequent appearance of VVC during pregnancy and increase of its recurrent form still are not clear. Among provoking factors of VVC local immunity and hormones disturbances are usually listed. However it is still a question which parameter of local immunity is most important and which factors are causal in the process of symbiotic to pathogenic flora and acute to chronic VVC transformation.

Antimicrobial peptide	Chemical features	Cells-producers; locuses	Known activity spectrum	Mechanism of action on *Candida* cells	Concentration provide *in vitro* activity against *C. albicans*	Concentration in vaginal fluid, μg/ml		Reference
						In healthy women	In patients with VVC	
	1	2	3	4	5	6	7	
Lactoferrin	Ferrum containing cationic glycoprotein, 76 – 80 kDa	Neutrophils (one of main peptides); secrets of endocrine glands, mucous membranes and human milk	Protozoa, fungi, bacteria, viruses	Exhaustion of ferric ions in medium; disruption of cytoplasmic membrane integrity; leakage of intracellular reserves of Ca(2+)	20 μg/ml	0.9 ± 0.2 μg/ml	no data	**2,3,4:** Salmon et al, 1997; Samaranayake 2001; Viejo-Diaz, 2004, van der Kraan, 2005 Lupetti et al, 2004 **5:** Bellamy et al, 1993 **6:** Valore et al , 2006
Calprotectine (=leucocyte protein L1)	Calcium and zinc – binding protein; 36,5 kDa, consist from 3 subunits with mol. mass 12,5 kDa	Form about 60% of protein fraction of neutrophils cytosol ; monocytes, macrophages of reactive tissues, squamous epithelium; blood plasma	Fungi and bacteria	Consumption of zinc from medium (competition in zinc ions)	4-32 μg/ml	5 -14 μg/ml	7 - 15 μg/ml	**2,3,5**: Brandtzaeg et al, 1995, **4:** Loomans et al,1998, Lulloff SJ, 2004 **6,7:** Valore et al, 2006
Lysozyme	Enzyme muramidase, 14,5 kDa	Monocytes, polymorphonuclear neutrophil; mucous membranes	Fungi and bacteria	Presumably damage of cell wall and cytoplasmic membrane	10 – 30 μg/ml	0.4 – 3 μg/ml	1.8 – 4.8 μg/ml	**2,3,4,5:** Ibrahim et al, 2001; Samaranayake et al, 1997 -2001 Marquis et al, 1982, 1993 **6,7:** Valore et al, 2006
secretory leukoprotease inhibitor (SLI)	Cationic nonglycosilated high-based acid-stable protein with high percent of cysteine, 11.7 kDa	Neutrophils, macrophages; c secrets of exocrine glands and mucous membranes	Fungi, bacteria, viruses	Presumably damage of cytoplasmic membrane; inhibition of microbial proteases	23 – 175 μg/ml	0,05-0,20 μg/ml	0,04-0,18 μg/ml	**1,2:** Doumas et al, 2005 Tomee et al, 1998; **5**: Tomee et al, 1997; **6,7:** Valore et al., 2006

Table 1. Continued

Antimicrobial peptide	Chemical features	Cells-producers; locuses	Known activity spectrum	Mechanism of action on *Candida* cells	Concentration provide *in vitro* activity against *C. albicans*	Concentration in vaginal fluid, µg/ml		Reference
						In healthy women	In patients with VVC	
	1	2	3	4	5	6	7	
cathelicidines	Amphypatic, cationic, alpha-spiraled peptides	Squamous epithelium and neutrophils of respiratory, gastrointestinal and urogenital tracts	Yeasts, bacteria	Destruction of cytoplasmic membrane				**1:** Moon et al. ,2006 **2,5:** Frohm et al, 1999 **3,4,5**: den Hertog et al, 2005 **6:** Valore et al., 2002
hCAP18	19 kDa				33 µg/ml	no data	no data	
LL-37	4,5 kDa				9 – 90 µg/ml	0,065 – 1,0	no data	
Defensins: **Beta:** HBD-1	Cationic nonglycosilated peptides with 6 cysteine residues, which form 3 intramolecular disulphide bridges of three-chain structure 3,5 – 4,5 kDa	Limphocytes; phagocytes; epitheliocytes of respiratory, gastrointestinal and urogenital tracts	Yeasts, bacteria and viruses	Membranes destruction	> 40 µg/ml	0,015- 0,035 µg/ml	0,010- 0,035 µg/ml	**2:** Taggart et al, 2003 **1:** Bals , 2000; Schneider et al, 2005; **6,7:** Valore et al, 2006 **5:** Feng et al, 2005; **5**: Cullor et al, 1990
HBD-2					4 µg/ml	0,005- 0,040	0,035-0,100	
Alpha: HNP-1-3					10 (only for HNP 1)	1,5 – 5	2.5 – 12.5	
HNP-5					50 µg/ml	0,007-0,025	0,005-0,025	

Table 1. Antimicrobial peptides of vaginal mucosa – biochemical properties.

From a quantity of factors of local immunity in VVC we can mark out few factors with direct antifungal activity: phagocytes (Nomanbhoy et al., 2002), antibodies (Barousse et al., 2004) and antimicrobial peptides (AMP) (Valore et al., 2002, 2006).

Phagocytosis of *C. albicans* is performed by neutrophyles and macrophages, that demonstrated by *in vitro* studies (Vonk et al., 2002). But there are no data on the action of these cells *in vivo*.

Among immunoglobulins most presented in vaginal secretions are IgG and secretory IgA. (Mestecky et al., 2005). But data concerning a relationship between immunoglobulins level and yeast population size are few a number and discrepant (de Carvalho et al., 2003; Kurnatowska et al., 2002; Mestecky et al., 2005).

However phagocytosis and immunoglobulins are common and relatively well investigated parameters of immunity whereas AMP as medical research subjects are insufficiently known.

In context of vaginal epithelium protection lactoferrin, calprotectin, lysozyme, leukoprotease secretory inhibitor, cathelicidins and defensins are mentioned.

The aim of the study was to determine a relationship between yeast population, severity of the disease and some parameters of local immunity in pregnant women with VVC.

3.1 Materials and methods

The study included 45 pregnant women aged 22 to 35 years old, conventionally divided into two main groups by the presence or absence of a chronic process. Each group was divided into two subgroups depending on the phase of the process at the time of the survey. Group has a name: RVVCE - women with recurrent VVC with exacerbation (n=9); RVVCR - women with recurrent VVC in remission (n=10); AVVC - women with primary acute VVC (n=13) ; ASYM - women with the minimum of typical symptoms of VVC at the time of the survey (n=13).

To assess the severity of the VVC the combination of the following symptoms was used: itching, burning sensation, the nature and amount of vaginal discharge, pain during urination, dyspareunia, dermatitis of perianal area, swelling, redness and erosive lesions of the vaginal walls. Each symptom was evaluated on a scale from 0 to 2, where a 0 means the absence of symptoms, 1 - moderate intensity, and for 2 - a vivid manifestation of a symptom.

Material for inoculation of medium was taken from the vaginal fornix with a sterile applicator, which was placed in a test tube with sterile transport medium «Amies» and delivered to the laboratory. Inoculation was carried out by the standard method on glucose-peptone-yeast growth medium containing the antibiotic.

Material for microscopy was collected with a sterile spatula from the vaginal fornix in a sterile container. A small amount (about 5 μl) sample was placed on a glass slide, pressed the coverslip so as to remove excess and to observe a monolayer of cells. Microscopy of samples were carried out at a total magnification of x1750. Efficiency of phagocytosis was evaluated as the ratio between the number of yeast cells, localized within the phagocytes, and the total number of yeast cells in the field of microscope.

Collection of vaginal secretions was carried out by the following method: put a tampon in the vagina «OB Pro comfort » of 10 minutes, after which the tampon was transferred to a plastic column 15x75 mm and eluted with 7 ml of distilled water using vacuum pump. The eluate was filtered through a bacterial membrane filter Millipore with a pore diameter of 0,22μ. The filtrate was freeze-dried and diluted in sterile distilled water so as to obtain a 10-fold concentrated relative to the initial filtrate (VF).

Immunoglobulins was assessed by dot-blot analysis . An antigen used in the analysis was obtained by selective extraction of surface proteins of cells *Candida albicans* (Arzumanian et al, 2000). Antigen at a concentration of 1mg/ml volume of 0.8 μl was applied onto a nitrocellulose membrane ("Whatman") with pore size 0.2 μ, dried and washed with 0,1% Tween-20 in saline, then with distilled water. Then incubated the membrane in the wells with 80 μl of blocking solution - PBS with 10% bovine serum - within 30 minutes, after which the well was added 20 μl of VF and incubated overnight. After incubation, liquid was removed, well washed, as described above, added 100 μl of conjugate solution in blocking solution. In determining the sIgA as a conjugate used mouse monoclonal antibodies to human sIgA conjugated with peroxidase at a dilution of 1:250; in determining IgG - mouse monoclonal antibody to human IgG conjugated with peroxidase at a dilution of 1:5000. After incubation for 1 hour the liquid was removed, the well with the membrane was washed as described above, added 100 μl of a solution containing hydrogen peroxide, TMB and precipitating agent, developed within 15-20 minutes. Results of the analysis were evaluated by 6-point scale from 0 to 5 depending on the intensity of the color spot.

Antifungal activity was determined as follows: two days old test culture of *Candida albicans* (№ 927, collection of Mechnikov Institute) were incubated with aliquots of VF with a temperature of 32^0 C and the ratio 20 μl of VF / 5 μl yeast suspension with concentration of cells 10^5 CFU / ml. From this mixture aliquots were inoculated on agar plates immediately after mixing and after 2 h of incubation. The result was expressed as the percentage of cells killed in the process of incubation.

3.2 Results and discussion

One of the aims of the study was to evaluate the local cellular antifungal immunity based on data fungal populations microscopy. We studied different of morphotypes data of fungal cells directly into the smears of women with VVC: single yeast cells (blastospores), pseudomycelium, true mycelium and blastospores enclosed in phagocytes. Summary data of microscopy of samples, culture tests and features of the VVC course in different groups of patients are presented in **table 1**.

The sum of symptoms characterizing the severity of theVVC for the group RVVCE varied from 5 to 13, whereas for the group RVVCR - from 1 to 9, for the group AVVC - from 2 to 8, for the group ASYM - from 1 to 6. Table 1 shows the median of these values. Obviously that this index correspond to the nature of the VVC. At the same time attention is drawn to the presence of symptoms in the group ASYM. The main contribution in this category belongs to the symptom of "the number and nature of vaginal discharge", namely, all patients in this subgroup had "milky" smears and in 38,5% of them there were abundant. Hereinafter we consider this category of patients as close to normal.

According to the established norm the increased contamination by yeast cells was observed in the two categories of patients – in AVVC and to a greater extent in RVVCE. However, despite the low seeding in the group RVVCR occurred the highest efficiency of phagocytosis (80%). This index was significantly lower in the acute phase, and in patients of ASYM group it was the lowest. Efficiency of phagocytosis in the subgroups varied as follows: in group ASYM from 0 to 50%, in AVVC - 20 ÷ 100%, in RVVCR - 50 ÷ 90%, and in RVVCE - 20 ÷ 100%. The frequency of detection of psevdomycelial cells was maximal in RVVCE and minimal in ASYM. Mycelium was detected much less frequently and only in subgroups with evidence of inflammation. Likewise divided within the groups frequency of positive cultural tests. Namely the presence of viable yeast cells in vaginal secretions in the group RVVCE was found in 100% of patients and varied in the range 1800 - 200,000 CFU/ml.

Compare all the above parameters with the sum of symptoms in the groups studied revealed the presence of a high degree of correlation of all indexes, except the efficiency of phagocytosis. In addition, the correlation coefficient between the frequency of positive cultural tests in the groups and medians of maximal number of yeast cells in the field of microscope was 0.921. There was no relationship between the efficiency of phagocytosis and yeast dissemination (r = 0,194), as well as between the efficiency of phagocytosis and the frequencies of positive cultural tests (r = - 0,134).

The next task of the study was to investigate the participation of local humoral immunity, namely immunoglobulins G and A. It is noteworthy that in all groups studied, more or less often the local IgG-antibodies to antigens of *C. albicans* were found. This is understandable, considering that none of the groups had patients who more or less would not be a carrier of opportunistic yeasts. However, in the acute phases of VVC was a significant increase in frequency of detection of IgG, especially in the group AVVC. The levels of these antibodies varied in all groups from 0 to 5, median values were: for RVVCE – 1, RVVCR – 0, AVVC - 2, ASYM - 0. Immunoglobulins of sIgA class did not occur in the patients group RVVCR, but at the exacerbation of a chronic process, these antibodies were detected in 25% of cases, while AVVC - 50% of cases. Interestingly, even in the group ASYM patients met with sIgA-antibodies. Levels of these immunoglobulins were varied as follows: for RVVCE- 0 to 3 (median 0), for RVVCR - 0; for AVVC - 0 to 3 (median 1), for ASYM - 0 to 2 (median 0).

The absolute values of the levels of IgG and sIgA did not correlate with either the abundance of yeast cells in vaginal secretions, or with frequency of positive cultural tests or with each other. Total of all 45 patients only 3 were detected the presence of both antibodies, while they were patients in the acute phase of the VVC. However, the detection rate of IgG-antibodies in the groups correlated with the medians of contamination of vaginal secretions (r = 0,772), whereas the frequency of detection of sIgA-antibodies was not associated with this index (r = 0,143).

The third objective of the study was determination of antifungal activity of antimicrobial peptides presented in vaginal secretions. We assessed the cumulative effect of AMP on the cells of *C. albicans* test culture (see "Materials and Methods"). It is important that the action of VF on yeast cells had a dose-depend character (**figure 1**). The highest activity against yeast cells possessed preparations obtained from vaginal secretions of ASYM group: spread of activity values was 34,4 ÷ 92,5%. In the group of patients with RVVCR this index ranged from 0 ÷ 63,8%; at RVVCE - 0 ÷ 48,4%; at AVVC - 0 ÷ 26,9%. Medians of these values are shown in table 1. Marked inverse correlation between the medians of antifungal activity and medians of following factors took place: the severity of the VVC (r = - 0,811), sowing

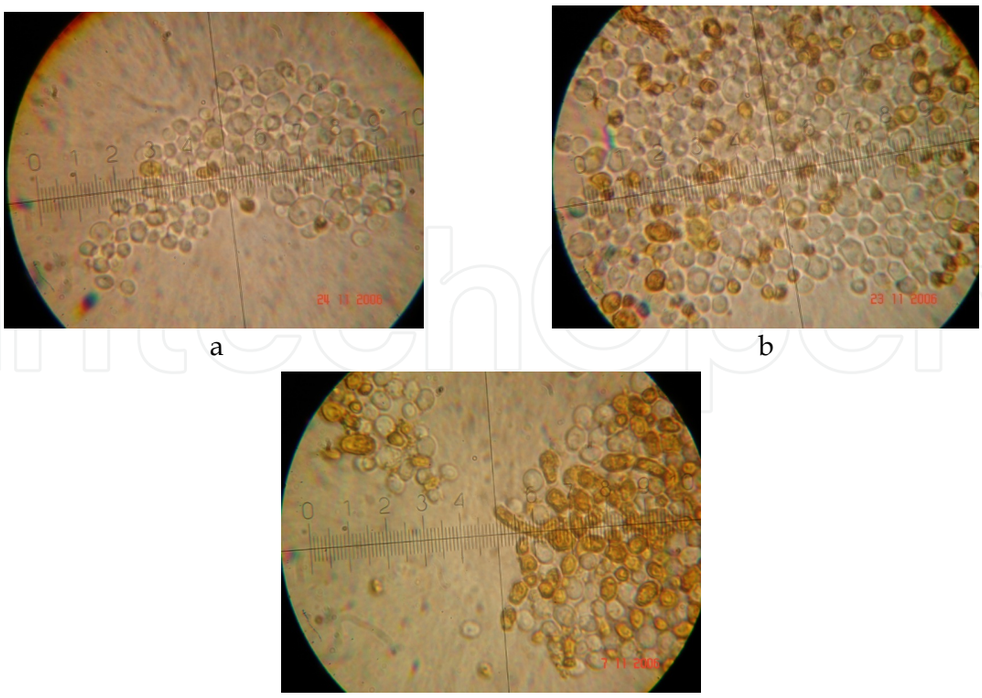

Fig. 1. Effect of different aliquots of vaginal fluid on the cells of *C. albicans* reference culture (stained by bromocresol purple); a - *C. albicans* incubated with 1 µl of vaginal fluid obtained from woman of ASYM group; b - 20 µl of vaginal fluid; c - 40 µl of vaginal fluid; alive cells – white, dead cells – yellow.

(r = - 0,689), contamination (r = - 0,855), IgG-antibodies (r = - 0,894), sIgA- antibodies (r = - 0,544). No correlation was found between the medians of antifungal activity and efficiency of phagocytosis (r = - 0,117).

Phagocytosis is an important mechanism of protection from opportunistic fungi. Traditionally an evaluation of phagocytosis and intracellular lysis *C. albicans* carry out by neutrophils and macrophages on *in vitro* models of cell cultures (Vonk et al., 2002). Revealed the presence of yeast blastospores not only in the intercellular space of vaginal smears, but within the phagocytes, we decided to estimate the ratio of the number of free and phagocyted yeast cells and use this index as an indicator of the efficiency of phagocytosis in this locus. Similar study with *C. albicans* test culture has previously been carried out (Valore E., 2006), but without analysis of correlation between VVC severity and antimicrobial activity of VF. According to the most recent data most presented in the vaginal secretions is immunoglobulin G (Mestecky et al, 2005). Attempts to establish the relationship between the severity of VVC and levels of immunoglobulins did not give unambiguous results (de Carvalho et al, 2003; Kurnatowska , Magnowski, 2002; Mestecky et al, 2005). Nevertheless, it is useful to compare the level and frequency of detection of secretory immunoglobulin in chronic and acute VVC with other parameters of local immunity.

Summarizing the data of correlation analysis of the studied parameters, we can conclude that for the category of patients, which is close to normal (ASYM), the most important part of local immunity is the total antifungal activity of soluble components of the vaginal secretions (**table**

1, 2). It is clear that at high antifungal activity of AMP the functioning of phagocytes and immunoglobulins is not necessary. Primary acute process (AVVC) caused by low antifungal activity, but it is characterized by significant activation of phagocytes and immunoglobulins. Chronization of acute process (RVVCE) also accompanied by a low antifungal activity and increased phagocytosis, but less frequent detection of immunoglobulins than in AVVC. Based on data of microscopy and cultural tests can be concluded that the acute phase of chronic VVC is always accompanied by the greatest severity of symptoms, an abundance of blastospores and often filamentous elements. These data are consistent with the results of studies conducted on large samples of patients with VVC (2861 pers.), where it is shown that the presence of hyphae of the mycelium is a marker of disease severity (Demirezen , Beksac , 2004). In the remission stage of chronic process (RVVCR) was noted the relatively high antifungal activity, a high level of efficiency of phagocytosis and the lowest level of immunoglobulins.

Group of patients	**n**	**Severity of VVC, (median)**	**Maximal amount of yeast cells in one field (median)**	**Frequency of positive cultural tests, %**	**Efficiency of phagocytosis, % of phagocyted cells in smears (median)**	**IgG, frequency of detection, %**	**sIgA, frequency of detection, %**	**Antifungal activity of VF, % of killed yeast cells (median)**
RVVCE	9	8	43	100	40	50	25	13,6
RVVCR	10	4	19	0	80	42,9	0	43,2
AVVC	13	5	30	30,8	50	62,5	50	12,5
ASYM	13	3	8	0	15	16,7	33,3	54,1
r*			***0,968***	***0,982***	***0,057***	***0,588***	***0,062***	***- 0,811***

* - correlation coefficient between certain index and severity of VVC

Table 2. Correlation between severity of VVC and data of microscopy of vaginal smears in different groups of patients

Thus, the direct antifungal activity of the soluble fraction of vaginal secretions, apparently, is the first condition, limiting the increase of fungal population. Reasons for the decrease of this activity may be, on the one hand, reducing the number of AMP, produced by neutrophils and epithelial cells, on the other - change the structure of AMP, leading to the loss of their activity. Human organism is forced to fill up the deficit of AMP through alternative mechanisms - activation of the local phagocytic function and increase the synthesis of specific secretory immunoglobulins. All these conclusions we summarized on the scheme (**table 3**).

GROUPS	**PHAGOCYTOSIS**	**IMMUNOGLOBULIN G**	**ANTIMICROBIAL PEPTIDES**
RVVCE	△	△	▼
RVVCR	△	△	△
AVVC	△	△	▼
ASYM	▼	▼	△

Table 3. Interrelation of investigated parameters of local immunity depending on form of VVC in pregnant women. ▼relatively low index; △ relatively high index

4. Bacterial vaginosis and the local antimicrobial activity in women

The colonization of female genital tract by alien microorganisms is determined by several factors, among which are normally listed the competition with resident microflora, exfoliation of the squamous epithelium, acidic and lactate rich medium. More than 20 years ago the antimicrobial peptides produced by polymorphonuclear neutrophils and epithelial cells were mentioning as a barrier in this locus (Cohen , 1984). Details concerning the role of each of the AMP, as well as contribute to the overall antimicrobial activity in vulvovaginal candidiasis mentioned above (**table 1**). In recent years a detailed study of the spectrum of these peptides in bacterial vaginosis (**BV**) and in the norm was carried out (Valore et al, 2002, 2006). In bacterial vaginosis was showed a reduction in the concentrations of AMP as compared with healthy women. The purpose of this study was to compare antimicrobial activity of vaginal discharge with the severity of bacterial vaginosis, as well as pH and some microbiological parameters.

4.1 Materials and methods

The study was conducted in 53 pregnant women, 44 of them - with bacterial vaginosis, and 9 - without it.

The severity of BV was evaluated by total score used a combination of the following symptoms: the nature and amount of bleeding, specific “fish” odor, pain when urinating, itching, burning, dyspareunia, dermatitis of perianal area, swelling, redness of the vaginal walls. Each symptom was evaluated on a scale from 0 to 2, where a 0 means the absence of symptoms, 1 - moderate intensity, and for 2 – vivid manifestation of a symptom. In accordance with the values of obtained scores all women was divided into subgroups: 1 - group consisted of women without signs of BV, then the subgroup is referred to as the "norm"; 2 - with mild BV (scores of 3 to 8 points); 3 - with moderate BV (9 to 12 points), 4 - with severe BV (13 to 18 points).

Material for microscopy taken with a sterile spatula from the vaginal fornix and placed on a glass slide. After staining the smears by Gram number of lactobacilli, bacteroides, gram-positive cocci and gardnerellas were estimated. Abundance expressed in points on four-point scale, where 0 - absence of this group of microorganisms, 1 - single cells in the visual field, 2 - moderate number of cells, 3 - an abundance of cells. Lactobacilli and bacteroides were combined into a group of "obligatory microflora”, and cocci and gardnerellas - a group of " facultative microflora”.

Collection of vaginal secretions (VF)was carried out as mentioned above (see chapter 3.1).
Total antimicrobial activity was determined as follows: cells of 4 days old test culture of *Escherichia coli* (№ 23, a collection of Mechnikov Institute) were incubated with aliquots of VF at a temperature of 32^0 C and a ratio of 40 µl of VF/ 10 µl of bacterial suspension density of 10^4 CFU / ml. Aliquotes of the mixture were seeded on Petri dishes with agar medium immediately after mixing and after 2 h of incubation. The result was expressed as the percentage of cells killed in the process of incubation.

Separation of proteins in samples was carried out by SDS-PAGE in 5-20% gradient polyacrylamide gel (Lambin et al., 1976). Samples were prepared in nondenaturating conditions by mixing 1 volume of sample with 2 volumes of buffer and causing the sample

to 40 µl per track. Staining was performed using Coomassie R-250. As molecular weight standards using a mixture of LMW ("Amersham Pharmacia").

Glucose was determined using VO meter "Accu-check Active" "Roche Diagnostics GmbH" (Germany). The result was expressed as mmol /l.

The pH was evaluated using indicator strips, intended for measuring range 3.8 - 6.0 ("LLC Lach-Ner", Czech Republic).

4.2 Results and discussion

Lactic-acid bacteria and bacteroids form a large part of the normal vaginal microflora (up to 10^8 CFU / ml), and are obligate microorganisms inhabiting this ecosystem. At the same time, the Gram-positive cocci, which are represented by entero-, strepto-, and staphylococci, are minor components of the vaginal microbiota (up to 10^3 CFU / ml), which can be distinguished by microscopic study. Even more rare and less desirable are the bacteria of the genus *Gardnerella*. Namely with increase in their population, and population of aerobic cocci is often linked BV. Therefore, the microorganisms identified by smear microscopy, we divided into two main groups - obligate (lactobacilli and bacteroides) and facultative (cocci and gardnerellas). Based on data of microscopy every smear was expessed in points. The results of the assessment in the form of arithmetic means are given in **table 4**. From the table it is followed that the highest scores on the obligatory microflora was observed in the absence of symptoms of BV. In part, index of facultative microorganisms were the lowest in the group "norm", and largest, respectively, in the groups with BV.

In general, in all groups the abundance of obligate microflora was in high inverse correlation relationship with abundance of facultative one (Pearson's correlation coefficient r = - 0,968). At that, the abundance of facultative microflora was directly correlated with the

Group of patients	**n**	**Severity of BV (medians)**	**Abundance of microflora, (medians)**		**Glucose (mmol/l) (medians)**	**pH (medians)**	**Antimicrobial activity of VF, % of killed *E. coli* cells (medians)**
			Obligatory	**Facultative**			
Norm (absence of BV symptoms)	9	0	6	1	1,9	4,4	78,2
Mild BV	12	8	3	5	1,7	5,0	36,4
Moderate BV	14	11	3	4	1,8	5,0	44,0
Severe BV	18	14	3	5	1,65	5,2	22,4
r*			**-0,914**	**0,885**	**- 0,830**	**0,975**	**- 0,944**

r- correlation coefficient between certain index and severity of BV

Table 4. Some biochemical and microbiological parameters of vaginal secretions in patients with BV and healthy women

severity of BV (***r*** = 0,885), but the abundance of obligate microflora - *vice versa* (***r*** = -0,914). Low pH of the vagina is reputed as the result of metabolism of lactic acid bacteria. Our data show that the lowest pH value had indeed taken place if there were not symptoms of BV and in the presence of a large number of lactobacilli. At the same time the biggest pH values were corresponded to severe form of BV. In other words, pH values were correlated not only with the severity of BV (r = 0,975), but with the nature of the microflora of this locus: between pH and obligate microflora relationship was characterized by ***r*** = - 0,962, and the pH and facultative microflora of ***r*** = 0,966.

As the concentration of glucose in vaginal secretions may affect the abundance of flora, we assessed the level of glucose in the studied groups of patients. It turned out that the greatest concentrations of glucose were detected in the absence of symptoms of BV, and the smallest - in the group with the severe form of the disease. There has been an inverse relationship of the index with the severity of BV (***r*** = - 0,830), a direct correlation with the abundance of obligate microflora (***r*** = 0,827), but the reverse - with abundance of facultative one (***r*** = - 0,933). Probably, the low level of glucose in the locus leaded to more intense amino acids consumption, which in part resulted in alkalization of the medium (pH increase) due to release of amines.

In determining the total antimicrobial activity resulting from the cumulative action of antimicrobial peptides, the highest index was appeared in a group of women without symptoms of BV, as well as the rise of symptoms was accompanied by decrease of antimicrobial activity (***r*** = - 0,944). Obviously, it is just reducing immune defense of locus resulted the increase of the opportunistic microflora population and the depletion of the normal microflora: the correlation between antimicrobial activity and obligate microflora was ***r*** = 0,926, while between antimicrobial activity and facultative microflora ***r*** = - 0,969. The division of VF proteins in a gradient of polyacrylamide gel demonstrated an association between antimicrobial activity and the presence / intensity of bands corresponded to antimicrobial peptides (**figure 2**). Track number 2 corresponds to the VF, obtained from women with mild BV (severity corresponded to 5 points, antimicrobial activity 100%). On this track, we can distinguish the following polypeptides - calprotectin with mol.mass about 37 kDa, cathelicidine hCAP18 (18 kD), secretory leucoprotease inhibitor – SLI (about 12 kDa), lysozyme (14.5 kDa) and defensin (less than 5 kDa). Track number 1 - the mild BV (severity score was equal to 7 points, antibacterial activity 66,7%) - no calprotectin, distinguishable other proteins, but less intense band. Track number 3 - severe BV (sum of symptoms 14, antimicrobial activity 21,6%) - no calprotectin and subtle defensin. Track number 4 - severe BV (total symptoms -17 points, antimicrobial activity 0%) - no calprotectin and defensin, a weaker band of lysozyme. Reduced concentrations of AMP in the VF of patients with BV have observed previously (Valore et al, 2006), however, the study of this phenomenon on a background of varying severity was carried out for the first time. From these data we can also conclude that the most important AMP in the antibacterial protection of the vagina in these patients are calprotectin, defensin and lysozyme.

Above, based on data from the literature, we compared the concentrations of AMP, which actually can inhibit the growth of *C. albicans*, with the levels of these substances in the vaginal secretions with the purpose of determine, which of these compounds are most important in the protection of this locus. It turned out that the most significant may be calprotectin, defensin HNP-1 and, to some extent, lysozyme. It is known that calprotectin causes depletion of the environment in trace elements, i.e. inhibits the growth of

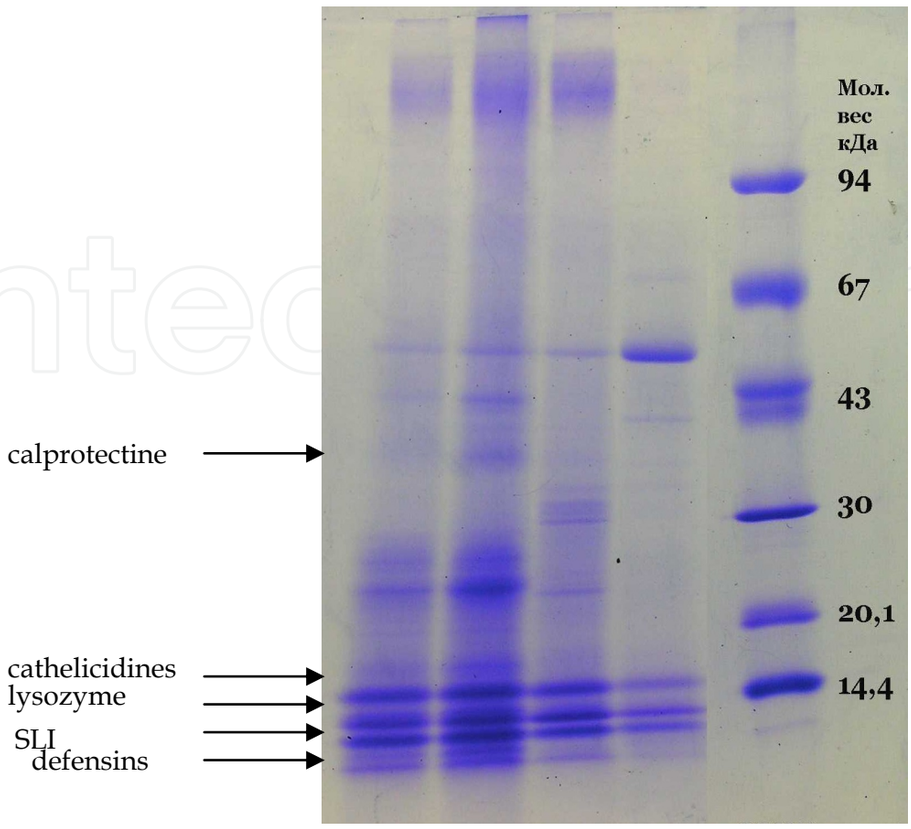

Fig. 2. SDS-PAGE of vaginal fluid samples obtained from patients with BV: track 1 - the mild BV (severity - 7 points, antibacterial activity - 66,7%track); track 2 - mild BV (severity - 5 points, antimicrobial activity - 100%); track 3 - severe BV (severity - 14, antimicrobial activity - 21,6%); track 4 - severe BV (severity -17 points, antimicrobial activity 0%); track 5 – mol.mass markers.

microorganisms (Loomans et al, 1998). Lysozyme destroys the glycoside bonds of polysaccharides of cell walls and damages the cytoplasmic membrane (Ibrahim et al, 2001). Defensin bound to negatively charged cytoplasmic membrane and cause the formation of pores (Schneider et al, 2005). Since these peptides were key in this study, we can conclude that in the mechanism of action they are not specific for microorganisms and act similarly to the pro-and eukaryotes.

Thus, we conclude that in pregnant patients with BV was noted a direct correlation between the severity of the disease and the level of pH of vaginal secretions, and invert correlation with the level of glucose in this locus. At the same time the high degree correlation between the BV severity and microbiological parameters was registered. Important role in the vaginal antimicrobial immune defense belongs to the total activity of AMP : calprotectin, defensins, lysozyme, cathelicidine and secretory leucoprotease inhibitor.

5. Antimicrobial peptides in local defence of skin

Surface epithelium of multicellular organisms is a barrier between the body and environment and works as active immune organ. Skin covered not only by several layers

of specialized cells, but antimicrobial substances – fatty acids, chloride ions and antimicrobial peptides. AMP are constitutively synthesized in normal epithelial cells and induced under the influence of several factors – microorganisms, disruption of skin integrity etc.

At last six classes of AMP – dermcidins, cathelicidins, defensins, ribonuclease 7, psoriasin and antileucoprotease – protect the skin from pathogenous and opportunistic microflora, with demonstrating of multiple functions concerned with immune defense.

Biochemical features of defensins, cathelicidins and secretory leucoprotease inhibitor we reviewed above (chapter 2). Below some properties of other peptides are listed.

Dermcidines were created in 2001 and shown to be secreted by merocrine sweat glands (Schittek et al, 2001). This class of peptides have the broad spectrum of action in the large diapason of pH and chloride ions ; molecular mass varied close to 5-8 kDa. They are excreted on the skin surface at 1-10 μg/ml of sweat, at that this concentration is toxic for bacteria and yeasts (Schittek et al, 2001; Flad et al, 2002). In connection with skin have mentioned the following dermcidins: DCD-1,DCD-1L, SSL-46, SSL-45, SSL-29, SSL-25, LEK-45, LEK-44, LEK-43, LEK-42, LEK-41, LEK-26, LEK-24, YDP-42, among which are cationic, anionic and neutral peptides. Interestingly that quantity of the secreted dermcidins depends on the certain body area: zones with intensive sweat excreting (axilla, hands, forehead etc.) had higher levels of dermcidins. Skin produces the peptides with the constant rate and they are stable during 72 hours. However in spite of their stability and broad spectrum of activity, some microorganisms are resistant to dermcidins. Presence of dermcidins is caused the production of specific proteases in *Staphylococcus aureus* and *S. epidermidis* (Lai et al, 2007). Estimation of synthetic dermcidin effect showed the absence of antifungal activity in this peptide (López-García et al, 2006).

Ubiquitous **ribonucleases** (RNKases) play important role in metabolism, angiogenesis, neurotoxicity, and antitumoral activity. Recently was created a new antimicrobial function of ribonucleases (Harder, Schroeder, 2002). The main source of RNKase 7 is keratinocytes. The peptide has molecular mass of 14,5 kDa, and destroys cytoplasmic membrane of different microorganisms even at low temperature (4 °C) and during some minutes (Huang et al, 2007). The lethal dose of RNKase 7 against vancomicin-resistant *Enterococcus faecium* was 30 nM.

Psoriasin (synonym S100A7) was known since the beginning of 90-th years as a peptide participated in inflammatory processes of chemotaxis, oncogenesis, angiogenesis and found in tissues of ear, skin, tongue and amniotic fluid (Madsen et al, 1991). It is the anionic peptide with molecular mass 11,4 kDa. High concentrations of the peptide contain the keratynocytes of patients with psoriasis, for which the infiltration of neutrophyls is typical. Antimicrobial activity of psoriasin was fixed in 2005 when the direct bactericidal action on *E. coli* was demonstrated by neutralization of the peptide by monoclonal antibodies (Glaser et al, 2005). The increase of psoriasin expression was found in presence of bacteria and proinflammatory cytokines , and decrease of the peptide activity took place in presence on zinc ions.

Apparently much in known about skin AMP, but for today no information exists concerning the native immune defense of skin appendages – hair and nails.

5.1 The congenital immune defence of hair

Skin appendages – nails and hair - are known to be affect by microorganisms with keratinase activity – dermatophytes, yeasts , rare staphylococci and propionic bacteria (Mikx, de Jong , 1987). However, not all people of the population are susceptible to these microbial agents, therefore apparently some defense of these loci must exist. Role of fatty acids and chloride ions in nail/hair protection is improbable, and so it remains to propose that AMP may realize the defense function.

Estimation of the presence and activity of AMP in normal hair keratinocytes was the aim of further study.

5.2 Materials and methods

Hair samples were obtained from 5 women 7 to 50 years old, which did not use the chemical coloration and hairdressing. Samples of fresh washed hair cut at a range of 10 cm from background. 360 mg of hair cut of scissors, then grinded by mortar and pestle up to homogeneity, adding drop by drop 8 ml 0,1 M solution of citric acid in 50% water ethanol [Harder, 2001]. The obtained cell-free homogenate was centrifuged during 7 min at the rate 10000 g, supernatant (about 3 ml) was dried at 27^0C in Petri dish. Dry extract was washed off by 0,5 ml potassium phosphate buffer ph 8,2 and centrifuged again; the final pH of solution was 6,5 (further we identify this solution as **E**). Control to E was the initial solution of citric acid processed by the same manner (**C**).

As a **test yeast culture** was used strain *Candida albicans* (№ 927 from collection of Mechnikov Institute) grown in glucose-pepton-yeast agar during 2 days at 27^0C.

For the study of **antimicrobial activity** we used 3 methods – determination of alive yeast cells by microscopy and inoculation, and estimation of growth inhibition zones. For microscopy 1 loop of culture was suspended in 1 ml of potassium phosphate buffer pH 4,6 , then 100 µl of the suspension was added to 80 µl solution E. After incubation during 2 hours at 32^0C, 800 µl of 2 mM bromocrezol purple solution in the same buffer was added (Kurzweilova H, Sigler K., 1993). Mixture was incubated during 1 hour at $32\ ^0$C, centrifuged at the rate 5000 g during 5 min, pellet microscoped at magnification x 1750 and photographed by camera Sony DSC-W7 (**figure 3**).

For the estimation of antimicrobial activity by inoculation method 1 loop of test-culture was suspended in 1 ml of sterile distilled water, and 10 µl of the suspension was added to 3 ml potassium phosphate buffer pH 5,5 (final cells concentration was approximately 10^3 CFU/ml). Then 20 µl of new suspension was added to 80 µl of solution E, mixed and inoculated the Petri dishes with agar immediately after mixing and after the certain time (**table 5**).

Dishes were incubated during 2 days at 27^0C, after that number of grown yeast colonies was calculated.

The zones of growth inhibition were estimated in the following way: warm molten agar was inoculated by test-culture cells (approximately 50 CFU/ml), filled in two Petri dishes and leave alone for congelation. After the agar surface drying, 20 µl of solution E and control solution C were put in the center of dishes (**figure 4**). Dishes were incubated during 4 days at 27^0C.

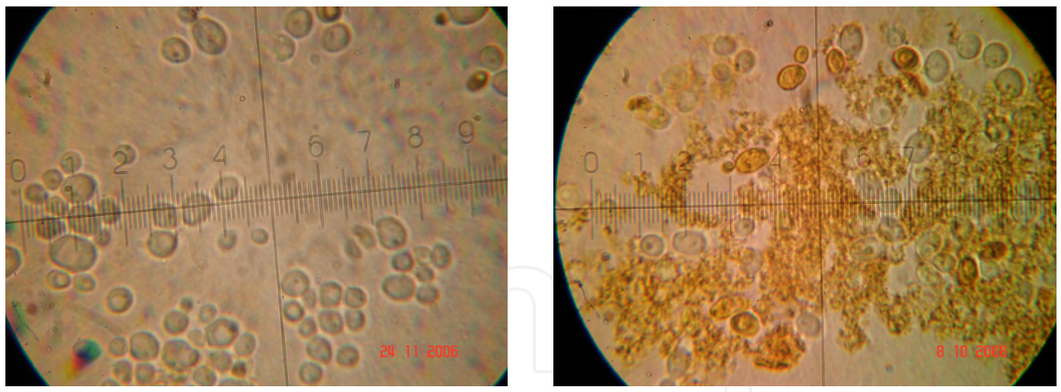

Fig. 3. Cells of *C. albicans* test-culture processed by extract of human hair : white cells – alive, dead and broken cells - yellow, dye – bromocrezol purple.

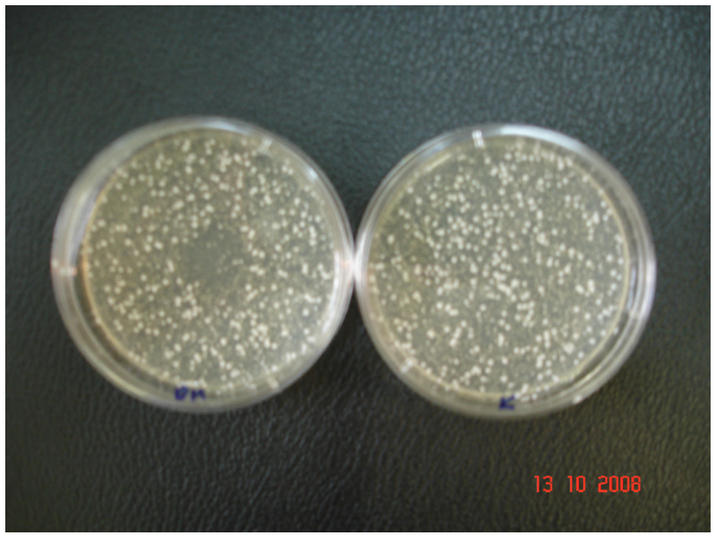

Fig. 4. Antimicrobial activity of hair extract against *C. albicans* test-culture : left dish is experimental, right one is control.

Time of exposition, hours	**% of killed cells, average means from 5 samples (5 experiments)**
0	0
0,5	20,7 ± 8,3
1	32,6 ± 7,3
3	46,7 ± 5,3

Table 5. Estimation of dieing rate of test-culture *C. albicans* cells under the incubation with hair extract.

Separation of proteins was carried out as written in chapter 4.1. Gels were stained by silver nitrate (**figure 5**). As the molecular mass standards LMW protein mixture was used ("Amersham-Pharmacia").

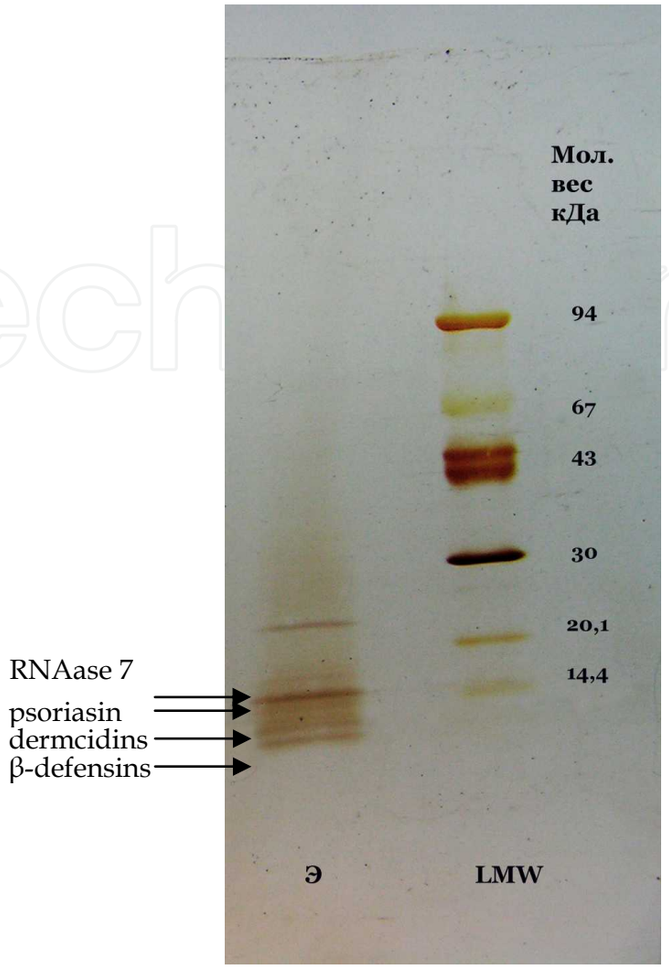

Fig. 5. Separation of proteins in gradient of SDS-PAGE: left track – hair extract, right track – low molecular weight markers.

5.3 Results and discussion

For the extraction of AMP from hair cells we used the solution, which was applied with the same purpose to the skin scales (Harder, Schroeder , 2001). The resulted extract even after centrifugation contained keratin – high molecular protein, the main component of keratinocytes. After neutralization of the extract by basic buffer the second centrifugation was necessary for the removing of keratin residue, which precipitated after the procedure.

Microscopy of test-culture cells processed by the solution E showed that alive cells were almost absent, whereas in the control sample all cells were alive (**figure 3**).

One can see large amount of cell debris, that is evidence of destruction of cell membranes typical for AMP, and disruption of cell walls. Such results did not enable to calculate the percent of killed cells.

The treatment of test-culture by hair extract and the following inoculation of agar showed, that during 3 hours a suspension with initial concentration about 200-300 alive cells in 20 μl,

lost about 52% of cells (**table 5**). At that cells, treated with control solution, completely saved alive.

Use of zone inhibition method demonstrated the presence of empty areola in the center of dish (diameter about 15-20 mm) with the absence of such one in the control (**figure 4**).

Thus we have proved the availability of antimicrobial activity in hair by 3 different ways. As the extraction method means the removal of peptides, it would be logically to expect a relationship between their presence and antimicrobial activity. Thereupon we separated the solution E by gradient SDS-PAGE (**figure 5**). It turned out that the extract really contained the low molecular proteins. Most distinct band corresponded to molecular mass about 14,4 kDa. Probably it is RNAase 7, which is usually expressed in skin keratinocytes (Harder, Schroeder , 2002). The second intensity had the the band with moleculad mass about 23 kDa, but among known skin AMP such peptides are absent. Multiple bands located in the diapason from 12 kDa to 3 kDa, they may correspond to psoriasin (11,4 kDa), dermcidins (5-8 kDa) and β-defensins (3,5-4,5 kDa).

Filtration of the extract through the membrane filter with pore diameter 3 kDa showed the absence of antimicrobial activity in the obtained solution.

From the data one can conclude that normal hear extracts displayed the antimicrobial action, which expressed in membrane lysis and disruption of cell walls. This antimicrobial activity is the result of AMP availability. Hair keratinocytes as skin keratinocytes contain the endogenous AMP, which play the important role in the innate defense of human hair from keratinophylic microorganisms.

6. Conclusion

Since the time of AMP creation much information were collected concerning their role in host defense against microbial agents. From our data and some facts from literature it is obvious that just low levels of these "natural antibiotics" in different loci are the reason of transformation of opportunistic microflora to pathogenic. Frequently even humoral and cellular components of immune system are secondary as compared with AMP: good example of this may be observed in vulvovaginal candidosis and bacterial vaginosis. In the innate defense of hair AMP also play the main role what is caused by the specific structure and "dry" consistence of this skin appendages. We may suppose that AMP should participate in the defense of nails.

The development of the investigation line was apparently concerned the working out of different substances similar to AMP as a new pharmaceutical antimicrobial preparations. Now on the base of knowledge about structure and function of AMP new antimicrobial preparations are developed (Bals, 2000). Synthetic and recombinant analogues of the peptides are at the study of pharmaceutical research, including clinical trials of I – III phase.

Lysozyme for example is already traditional preparation used in the cases of local microbial affection. It is possible that the analogues of natural AMP will have not only antimicrobial activity, but could be used as immunomodulators.

The other direction of research may be study of stimulation of AMP synthesis *in vivo* in immune cells – neutrophils, epitheliocytes etc. – by immunomodulating agents. There are

many such preparations in pharmaceutical market, which are known to have the stimulating effect on the proliferation of immune cells. However it is still a question if they may increase the AMP synthesis or not.

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